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Prostate cancer (PCa) is a leading cause of death in men in the United States (1). If detected early when the tumor is still confined to the prostate, there are a number of treatment regimens that lead to good prognosis. However, if the tumor escapes the prostate prior to diagnosis, patient prognosis is poor. The objective of the proposed study was to identify serine hydrolases that are aberrantly regulated in PCa and that contribute to progression of the disease. To accomplish this objective we undertook a unique approach called activity-based protein profiling. In this method, a chemical probe composed of a "warhead" and a detection reagent are used to covalently tag the active site of an enzyme. Using this strategy we have identified a number of novel serine hydrolases that are expressed in prostate cancer. Of special importance is an enzyme called fatty acid synthase, which contains a serine hydrolase domain. We identified a lead inhibitor of this domain of fatty acid synthase, called Orlistat, which is a drug approved for treating obesity. Our work also shows that Orlistat can slow the growth of prostate tumors in mouse models of human PCa.

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# Table of Contents

Introduction

Prostate cancer (PCa) is a leading cause of death in men in the United States (1). If detected early, when the tumor is still confined to the prostate, there are a number of treatment regimens that lead to good prognosis. However, if the tumor escapes the prostate prior to diagnosis, patient prognosis is poor. The objective of the proposed study was to identify serine hydrolases that are aberrantly regulated in PCa, and that contribute to progression of the disease. We used a novel proteomics strategy called activity-based protein profiling (2) to identify enzymes that exhibit aberrant activity in prostate cancer. One of the enzymes we identified is fatty acid synthase. Fatty acid synthase is the sole enzyme responsible for the cellular synthesis of fatty acids. This enzyme has been previously linked to tumor growth (3, 4), so screens were established to identify novel antagonists of this enzyme. Surprisingly, one of the more potent antagonists that was identified is tetrahydrolipstatin, also called Orlistat. This compound is approved by the FDA for treating obesity. Further work showed that Orlistat selectively inhibits tumor cell growth and induces tumor cell apoptosis. More significantly, Orlistat slows the growth of prostate tumors in mouse models of prostate cancer.


Body

The initial goal of the proposed study was to identify enzymes that are involved in the progression of prostate cancer. We used an activity-based proteomics probe to identify serine hydrolases that showed activity in prostate cancer. The chemical probe that we used, based on a fluorophosphonate electrophile, reacts covalently with the active site serine residue of all serine hydrolases. This enzyme family is composed of enzymes with a wide array of functions and includes serine proteases, lipases, and thioesterases and carbylesterases.

With this probe, we profiled serine hydrolase activity in the mouse TRAMP model and in a panel of human prostate cancer cell lines. One serine hydrolase whose activity is consistently up-regulated in the prostate cancer samples is fatty acid synthase (FAS). This large enzyme consists of six enzymatic pockets and an acyl carrier protein. Together these pockets synthesize palmitate, the primary cellular precursor for all fatty acids. The last enzymatic pocket of fatty acid synthase is a thioesterase, which has the serine hydrolase architecture and which is hit by the activity-based probe.

Thus, with the identification of fatty acid synthase as being aberrantly regulated in PCa, we satisfied the objectives of the Statement of Work for Year 1.

A second objective of the study was to obtain neutralizing antibodies against the targets that we identified in PCa, and to use them to determine if the enzymes have a causal role in the progression of PCa. Because fatty acid synthase is expressed within the cytoplasm, we reasoned that antibodies would not be valuable antagonists for probing its function, so instead we screened for small molecule antagonists of fatty acid synthase. Surprisingly, we identified Orlistat as an inhibitor of FAS. Orlistat (tetrahydrolipstatin) is an FDA-approved drug approved for treating obesity (Xenical). Its stated molecular target is pancreatic lipase, not FAS.

Our studies also show that inhibition of FAS by Orlistat will induce cell-cycle arrest, thereby halting tumor cell proliferation. Most significantly, we have discovered that Orlistat is able to slow the growth of human xenograft prostate tumors growing in nude mice. A daily dose of 175mg/kg of Orlistat delivered by i.p. injection slowed the growth of PC-3 tumors by more than 60%.

These findings validate fatty acid synthase as a drug target for PCa and are described in our paper entitled "Orlistat Is a Novel Inhibitor of Fatty Acid Synthase with Antitumor Activity," which was published in *Cancer Research* (manuscript included with this report). Since we identified and validated fatty acid synthase as a useful therapeutic target in PCa, we have also satisfied the Milestones stated for Years 2 and 3 of the project.

Our work has also led to a mechanistic understanding of how fatty acid synthase ties into the regulation of the cell cycle in tumors. In a paper that we recently published in the *J. Biol. Chem.* entitled "A Fatty Acid Synthase Blockade Induces Tumor Cell-cycle Arrest by Down-regulating Skp2," which is attached to this report, we show that the blockade of fatty acid synthase elicits a blockade at the $G_1/S$ checkpoint by up-regulating a protein called S-phase kinase associated protein (skp)-2. These findings provide important mechanistic insight into the role of fatty acid synthase in tumor progression and show that our observations on PCa are also relevant to breast cancer. Unpublished work from our group also indicates that fatty acid synthase blockade is cytostatic and cytotoxic to tumor cells derived from the ovaries and the colon. Therefore, fatty acid synthase is likely to be a valid therapeutic target for all solid tumors.


Key Research Accomplishments

- An initial identification of several of the serine hydrolases present in prostates of TRAMP mice was performed, revealing fatty acid synthase as an enzyme that is frequently up-regulated in prostate tumors.
- Parallel profiling studies were performed on normal and neoplastic human prostate cells. These comparison also show fatty acid synthase to be up-regulated in the tumor cells.
- An initial screen for inhibitors of serine hydrolases in prostate cancer lines has been completed. A surprising finding of this screen was the identification of Orlistat (an FDA-approved drug) as an inhibitor of fatty acid synthase.
- The effects of Orlistat on normal and neoplastic prostate cancer cells has been tested, revealing that this drug induces a $G_1/S$ cell-cycle arrest and in several instances will cause tumor cell selective apoptosis.
- Orlistat is able to block prostate tumor growth *in vivo*. Administration of Orlistat to mice bearing human PC-3 prostate xenografts blocks tumor growth. This observation validates FAS as an antitumor target and also puts Orlistat forward as a lead molecule for drug development.
- We find that a primary effect of fatty acid synthase blockade on tumor cells is the up-regulation of a key cell-cycle regulatory protein called S-phase kinase associated protein (skp)-2.
- Fatty acid synthase appears to be a valid target in the major classes of solid tumors, including prostate, breast, ovarian, and colon.

Reportable Outcomes

- This study has led to the publication of two manuscripts, which are attached to this report.

  Steven J. Kridel, Fumiko Axelrod, Natasha Rozenkrantz, and Jeffrey W. Smith. (2004). Orlistat Is a Novel Inhibitor of Fatty Acid Synthase with Antitumor Activity. Cancer Res. 64, 2070–2075.

  Lynn M. Knowles, Fumiko Axelrod, Cecille D. Browne, and Jeffrey W. Smith. (2004). A Fatty Acid Synthase Blockade Induces Tumor Cell-Cycle Arrest by Down-Regulating Skp2. J. Biol. Chem. 279, 30540–30545.

- One patent application entitled "Inhibition of Fatty Acid Synthase by Beta-Lactones and other Compounds for Inhibition of Cellular Proliferation." has been filed based on the work performed under this award.
- One NIH grant application has been funded based on work performed under this award (CA 106582: Novel Anticancer Fatty Acid Synthase Beta-Lactone Inhibitors). The objective of this grant is to identify more potent and selective inhibitors of fatty acid synthase as antitumor agents. The grant is a collaboration between Dr. Daniel Romo (Texas A&M) and Dr. Jeffrey W. Smith (Burnham).

Conclusions

The primary conclusions of the study are:

1) Fatty acid synthase is a valid target for antitumor therapy in prostate and other cancers.

2) Antagonists of fatty acid synthase induce a $G_1/S$ arrest in tumor cells and elicit tumor-selective apoptosis.

3) Antagonists of fatty acid synthase inhibit the growth of tumors in animal models of human prostate cancer.

4) The FDA-approved drug Orlistat (tetrahydrolipstatin) is a novel inhibitor of the fatty acid synthase thioesterase and is able to induce $G_1/S$ cell-cycle arrest and apoptosis in prostate cancer cells and inhibit the growth of prostate tumors in animals.

So What? This study illustrates that fatty acid synthase is a rationale therapeutic target in prostate cancer, and provides a lead compound for drug development.

Citations

1.      Small, E. J. Advances in prostate cancer. Current Opinion in Oncology, *11:* 226-235, 1999.

2.      Liu, Y., Patricelli, M. P., and Cravatt, B. F. Activity-based protein profiling: the serine hydrolases. Proceedings of the National Academy of Sciences of the United States of America, *96:* 14694-14699, 1999.

3.      Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A., and Han, W. F. Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. Cancer Research, *58:* 4611-4615, 1998.

4.      Pizer, E. S., Pflug, B. R., Bova, G. S., Han, W. F., Udan, M. S., and Nelson, J. B. Increased fatty acid synthase as a therapeutic target in androgen-independent prostate cancer progression. Prostate, *47:* 102-110, 2001.

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# Appendices

# Orlistat Is a Novel Inhibitor of Fatty Acid Synthase with Antitumor Activity

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## ABSTRACT

One of the fundamental principles of pharmacology is that most drugs have side effects. Although considerable attention is paid to detrimental side effects, drugs can also have beneficial side effects. Given the time and expense of drug development, it would be particularly exciting if a systematic method could be applied to reveal all of the activities, including the unappreciated actions, of a potential drug. The present study takes the first step along this path. An activity-based proteomics strategy was used to simultaneously identify targets and screen for their inhibitors in prostate cancer. Orlistat, a Food and Drug Administration-approved drug used for treating obesity, was included in this screen. Surprisingly, we find a new molecular target and a potential new application for Orlistat. Orlistat is a novel inhibitor of the thioesterase domain of fatty acid synthase, an enzyme strongly linked to tumor progression. By virtue of its ability to inhibit fatty acid synthase, Orlistat halts tumor cell proliferation, induces tumor cell apoptosis, and inhibits the growth of PC-3 tumors in nude mice.

## INTRODUCTION

Most drugs have side effects. These range in magnitude from simple nuisances to life-threatening complications. Although focus is placed on negative side effects, beneficial side effects are also observed. Unfortunately, the unanticipated effects of a drug are often revealed in the later stages of development or even after the drug has been approved for use. Given the time and expense of drug development, it would be particularly exciting if all activities of a compound could be revealed at the outset of its development. With such information, care could be taken to minimize detrimental side effects, and testing of the drug could be expanded to other indications should its activity profile warrant.

The ability to perform all of the encompassing screens of the activity of a drug may be on the horizon. In principle, one could predict the effects of a drug by knowing all of its targets. Recent emphasis on global profiling strategies, including gene expression profiling and proteomics, drives this type of thinking (1). Yet these profiling technologies measure abundance, not function, and they fall short of making it possible to screen drugs against a plethora of targets. Recent work in the area of chemical biology points the way toward direct profiling of protein activity, offering a possible solution to this hurdle. Two groups have created chemical probes that react at the active site of multiple enzymes of a given class. Liu *et al.* (2) synthesized a probe containing fluorophosphonate as the warhead and biotin as the reporter, and then used this probe to reveal the serine hydrolase activity profile in biological samples. Greenbaum *et al.* (3) showed that the cysteine proteinases profile could be visualized with probes containing reactive epoxides. Because activity-based probes bind at an active site of the enzyme, a direct measure of the level of active enzyme can be obtained. Consequently, it becomes possible to use straightforward competition assays to screen for inhibitors of all of the enzymes within a family.

Here we apply the activity-based screening strategy to identify serine hydrolases in prostate cancer cells. The activity-based nature of the screen also allows us to identify inhibitors of these enzymes. Of particular interest is fatty acid synthase (FAS), which is up-regulated in the prostate cancer (PCa) cells compared with normal prostate epithelial cells, and has been implicated in the progression of various types of tumors (4–9). Interestingly, Orlistat is a novel and rather selective inhibitor of FAS in tumor cells. This drug inhibits the thioesterase function of the enzyme, interferes with cellular fatty acid synthesis, and can halt tumor cell proliferation and induce tumor cell apoptosis. Orlistat also inhibits the growth of PC-3 prostate tumors *in vivo*. Altogether the study reaffirms the significance of FAS in tumor progression and underscores the fact that this enzyme is a valid oncology target. The study also indicates that compounds with reactive $\beta$-lactones, such as Orlistat, should be evaluated as potential antitumor agents.

## MATERIALS AND METHODS

**Activity Profiling of Serine Hydrolases.** LNCaP, DU-145, and PC-3 cell lines (American Type Culture Collection) were maintained in RPMI 1640 (Irvine Scientific) supplemented with 10% fetal bovine serum at 37°C in 5% $CO_2$. The PrEC cell line (Clonetics) was maintained in defined medium supplied by Clonetics. Each cell line was maintained in 150-mm tissue culture dishes. To generate protein lysates, cells were washed with ice-cold PBS and harvested by scraping with a cell lifter into cold PBS. Cells were collected by centrifugation, resuspended in 50 mM Tris-Cl (pH 8.0), and then lysed by sonication as described previously (2, 10). Soluble and insoluble cell fractions were separated by ultracentrifugation for 1 h at 64,000 rpm at 4°C. Protein concentrations were determined by BCA assay (Pierce).

Activity profiling was performed with fluorophosphonate (FP)-polyethylene glycol (PEG)-6-carboxytetramethylrhodamine (TAMRA) using methods described previously (2, 10). Briefly, soluble fractions (40 $\mu$l; 1 mg/ml) were treated with 2 $\mu$M FP-PEG-TAMRA for 1 h at ambient temperature. Reactions were stopped by the addition of Laemmli buffer and boiling. Nonspecific reaction of the probe was determined with a duplicate sample boiled for 10 min before labeling with FP-PEG-TAMRA. The labeled samples were resolved by 10% SDS-PAGE and visualized by scanning with a Hitachi flatbed scanner at 605 nm.

Serine hydrolase activity in whole cells was measured with a membrane-permeable probe, FP-BODIPY. After addition of Orlistat, the probe was added to cells (final concentration of 2 $\mu$M), and the reaction was allowed to proceed to completion (1 h). Cells were lysed by the addition of Laemmli sample buffer and boiled; samples were resolved on SDS-PAGE and visualized by scanning with a Hitachi flatbed scanner at 605 nm.

**Inhibition of Serine Hydrolase Activity by $\beta$-Lactones.** Ebelactone A and B stocks were made in DMSO. Orlistat (Roche) was solubilized from pills in absolute ethanol. Cell lysates were generated at 1 mg/ml as described above. Samples (40 $\mu$g) were incubated with inhibitors for 20 min, and FP-PEG-TAMRA was added and reacted for an additional 30 min.

**Identification of Labeled Serine Hydrolases.** To identify serine hydrolases, a fluorophosphonate probe linked to biotin was used (2, 10). Cell lysates were preadsorbed to avidin-agarose to reduce nonspecific binding of proteins during the purification. Cell lysates were labeled with FP-PEG-biotin (5 $\mu$M) for 1 h at room temperature. Protein was separated from unincorporated FP-PEG-biotin by gel filtration on Nap 25 columns. SDS was added to the eluate to a concentration of 0.5%, and the sample was denatured by boiling. Samples were diluted with 50 mM Tris (pH 7.5) and 150 mM NaCl, and

incubated with avidin-agarose for 1 h at room temperature. The agarose beads were washed eight times with 50 mM Tris (pH 7.5) and 150 mM NaCl containing 1% Tween 20. Labeled protein was eluted with Laemmli buffer containing 1% SDS. Protein was resolved by 10% SDS-PAGE and detected by silver staining. Specific bands were extracted and subjected to in-gel digestion by trypsin and peptide mass fingerprinting with matrix-assisted desorption ionization-time of flight as described previously (11, 12).

**Expression of the Recombinant Thioesterase Domain of Fatty Acid Synthase.** The portion of the FAS gene (gi:21618359) encoding the thioesterase domain was amplified by PCR using the following primers: 5′ ATG ACG CCC AAG GAG GAT GGT CTG GCC CAG CAG (corresponds to nucleotides 6727–6756) and 3′ GCC CTC CCG CAC GCT CAC GCG TGG CT (corresponds to nucleotides 7625–7650). The recombinant thioesterase domain was cloned into pTrcHis (Invitrogen) and expressed in *Escheria coli*. The recombinant protein corresponds to residues 2202 through 2509 of FAS. The thioesterase was purified by Ni-affinity chromatography, and analyzed for activity and inhibition by Orlistat, using methods described above.

**Detection of Fatty Acid Synthase by Western Blot.** PC-3 cells ($5 \times 10^4$) treated with Orlistat were boiled in Laemmli buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. The membrane was blocked with nonfat milk and probed with an anti-FAS monoclonal antibody (PharMingen). Binding was visualized with an horseradish peroxidase-conjugated rabbit antimouse IgG (Bio-Rad) followed by chemiluminescent detection with the Western Lighting Chemiluminescence Reagent (Perkin-Elmer).

**Inhibition of Fatty Acid Synthesis by Orlistat.** Cellular fatty acid synthesis was measured by the incorporation of [$^{14}$C]acetate (13, 14). Cells ($2.5 \times 10^4$ cells/well in 24-well plates) were washed twice with PBS and incubated in defined serum-free medium containing 300 $\mu$g/ml BSA and insulin, transferrin, and selenium as supplements. Medium was added to the cells in the presence or absence of Orlistat. Cells were incubated with Orlistat for up to 2 h before the addition of 1 $\mu$Ci of [$^{14}$C]acetate. Cells were incubated with [$^{14}$C]acetate for 2 h, at which time medium was removed, and the cells were washed with PBS/EDTA and trypsinized. Cell pellets were washed twice more with PBS, and fatty acids were extracted with chloroform-methanol (1:1) for 30 min. The extract was dried under $N_2$ and extracted with water-saturated butanol. Butanol was evaporated under $N_2$, and labeled fatty acids were detected by scintillation counting.

**Effects of Orlistat on Cell Proliferation.** PC-3 cells were exposed to Orlistat along with different concentrations of palmitate for 48 h. Fresh medium, along with Orlistat and palmitate, were added every 24 h. Prolifer-
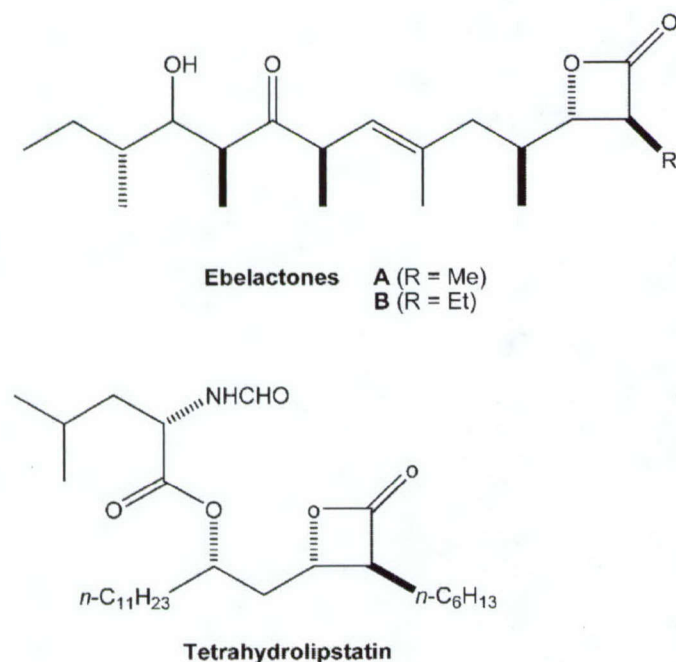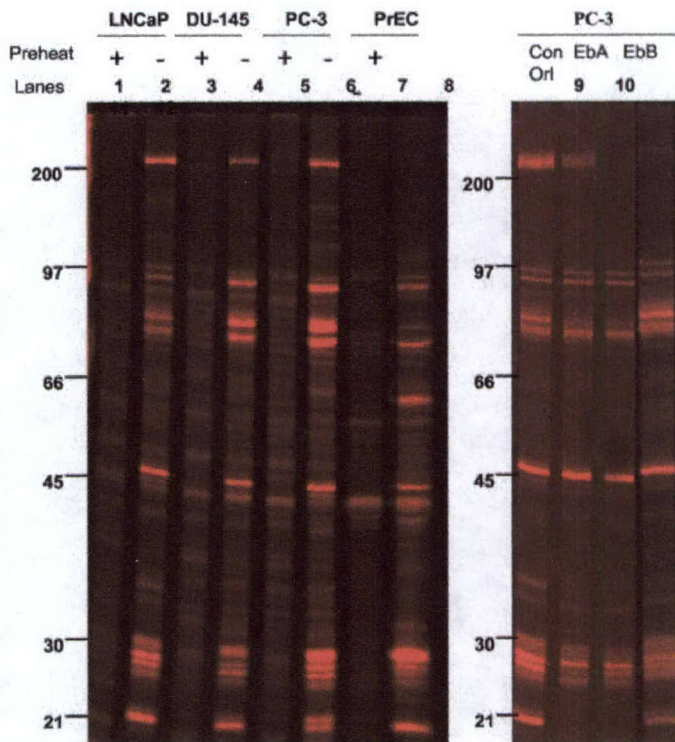


Fig. 2. Activity profiling of normal and neoplastic prostate epithelial cells. Lysates were generated from primary cultures of normal prostatic epithelial cells (*PrECs*) and from three prostate tumor cell lines (*LNCaP, DU-145,* and *PC-3*). Lysates were incubated with fluorophosphonate-polyethylene glycol-6-carboxytetramethylrhodamine (FP-PEG-TAMRA) for 1 h at room temperature. Nonspecific labeling with the activity probe was measured in samples denatured by boiling (lanes marked +). Samples were resolved by 10% SDS-PAGE and visualized at 605 nm using a Hitachi flatbed gel scanner (*lanes 1–8*). The effect of three $\beta$-lactones on the activity labeling of serine hydrolases from prostate cancer cells was assessed in a similar manner. Before incubation with FP-PEG-TAMRA, lysates were preincubated with ebelactone A (*lane 10*), ebelactone B (*lane 11*), or Orlistat (*lane 12*). After labeling with FP-PEG-TAMRA, the reactions were halted and enzyme activity visualized as described above.

ation was assessed by measuring bromodeoxyuridine labeling using the Cell Proliferation ELISA (Roche).

**Effects of Orlistat on Cell Death.** Cells were plated in 96-well tissue culture plates in complete medium. After 24 h, the cells were exposed to Orlistat for an additional 24 h. Apoptosis was measured with the Cell Death Detection ELISA (Roche), which was performed according to the manufacturer's protocol. As an independent assessment of apoptosis, the amount of cleaved poly(ADP-ribose) polymerase was measured in cells after treatment with Orlistat. Cells were cultured with the Orlistat (25 $\mu$M) or ethanol for 72 h, or with Staurosporine (1 $\mu$M) for 5 h. At each time point, total cell extracts were generated by addition of 1× SDS sample buffer. Samples were subjected to Western analysis using antibodies against the cleaved form of poly(ADP-ribose) polymerase (Cell Signaling). Western blotting was performed according to protocols established by the manufacturer of the anti-poly(ADP-ribose) polymerase antibody

**PC-3 Xenograft Tumor Model.** The effect of Orlistat on growth of PC-3 tumors in nude mice was assessed with a staged model. PC-3 cells ($1 \times 10^6$) were injected into the flank of male athymic nude mice 4–5 weeks of age. Tumors were allowed to grow until they reached a size of ~100 mm$^3$, at which time Orlistat administration was initiated. Orlistat was administered in 30 $\mu$l of vehicle containing 33% ethanol and 66% PEG 400. Animals received 240 mg/kg/day of Orlistat. Tumor size was measured with calipers twice weekly, and volume was calculated with the formula volume = $\pi/6 \times XY^2$ (15).

## RESULTS AND DISCUSSION

An activity-based proteomics screen was used to identify serine hydrolases in PCa cells and to screen for their inhibitors. Serine hydrolases were revealed with an activity-based probe composed of a



**Ebelactones**   A (R = Me)
                  B (R = Et)



**Tetrahydrolipstatin**

Fig. 1. Structures of $\beta$ lactones.

FP warhead linked to the fluorophore (FP-TAMRA) (16). Primary cultures of normal prostate epithelial cells (PrEC) were compared with three PCa cell lines, LNCaP, DU-145, and PC-3. Cell lysates were reacted with FP-TAMRA and then resolved on SDS-PAGE (Fig. 2, *lanes 1–8*). In each case, ~15 different hydrolases were detected as fluorescent bands on SDS gels. The pattern of serine hydrolase expression is generally similar among the cell lines, with two significant distinctions. A band of 62 kDa was active in the normal PrECs but absent in all of the tumor lines. Peptide mass fingerprinting showed this enzyme to be carboxylesterase-2. Conversely, a hydrolase with a mass of ~270 kDa is expressed in all of the tumor lines but absent in normal PrECs. Peptide mass fingerprinting with mass spectrometry showed this band to be FAS, an observation that was confirmed by immunoprecipitating the complex between FP-TAMRA and FAS (Fig. 3*B*).

FAS is responsible for the conversion of dietary carbohydrate to fat and is the only eukaryotic enzyme capable of synthesizing palmitate, the precursor for the majority of nonessential fatty acids (17). FAS has a unique structure and mode of action. The enzyme contains seven separate enzymatic pockets and an acyl carrier protein. The distinct enzyme domains of FAS operate together to condense acetyl CoA and malonyl CoA, ultimately generating the 16 carbon polyunsaturated fatty acid palmitate. Palmitate remains covalently attached to the acyl carrier protein of the enzyme until it is liberated by the final enzymatic pocket on the enzyme, the intrinsic thioesterase. This thioesterase is the sole serine hydrolase within FAS and is the target of the FP-TAMRA probe (see below).
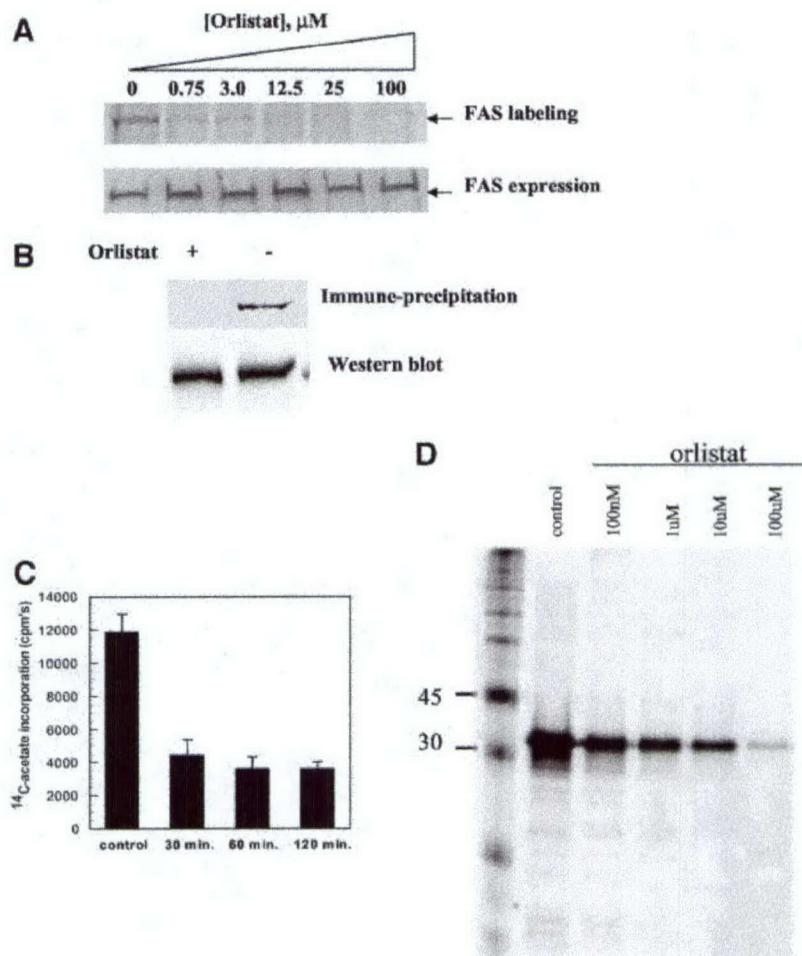
FAS is up-regulated in many tumors. Its function has been strongly linked to tumor cell proliferation (18), making it an attractive therapeutic target. A functional connection between FAS and tumor cell proliferation was originally suggested by work with the fungal product cerulenin and its synthetic derivative c75. These compounds inhibit the ketoacyl synthase domain of FAS, the first enzymatic pocket in the enzyme (19, 20), and have shown some promise as antitumor agents (8, 20–22).

We capitalized on the fact that FP-TAMRA reacts with the active site of the thioesterase domain of FAS to screen for alternative inhibitors of this enzyme. Three derivatives of natural products, each containing a $\beta$-lactone moiety, were tested for the ability to block activity-based labeling of FAS. These are ebelactones A and B, and tetrahydrolipstatin, which is also known as Orlistat and is marketed as Xenical (Fig. 1). The $\beta$-lactone can undergo nucleophilic attack on the carbonyl carbon of the lactone ring by the active site serine of the esterase, yielding a covalent adduct between enzyme and inhibitor (23). All three of the compounds inhibit the thioesterase of FAS (Fig. 2, *right panel, lanes 10–12*), but only Orlistat is selective for FAS in tumor cells. Orlistat is a drug approved and widely used for weight management in obese patients (24). The effectiveness of Orlistat in obesity is conferred by the ability of the drug to inhibit pancreatic lipase in the gastrointestinal tract, thereby preventing uptake of dietary fat. The inhibition of FAS by Orlistat has never been reported and is not believed to be relevant to its mode of action in weight loss.

To characterize the effects of Orlistat on FAS in intact cells, we measured the ability of the compound to inhibit the activity of FAS in whole cells. PC-3 cells were treated with a range of Orlistat, and the level of FAS thioesterase function was measured with a membrane-



Fig. 3. Effect of Orlistat on cellular activity of fatty acid synthase (*FAS*). *A*, FAS activity in intact PC-3 cells was measured with a membrane-permeable activity probe, fluorophosphonate (FP)-BODIPY. PC-3 cells were first incubated with a range of Orlistat, and the remaining FAS activity was assessed by adding FP-BODIPY to the cells. Cell lysates were separated on SDS-PAGE, and active serine hydrolases were visualized (*top panel*) as described in "Materials and Methods." The effect of Orlistat on the level of FAS protein was measured using the same cell lysates as in by Western blot (*bottom panel*). *B*, immune precipitation of the complex between FAS and FP-6-carboxytetramethylrhodamine (TAMRA). Cell lysates were treated with 12 $\mu$M Orlistat (+) or vehicle control (−) before labeling with FP-TAMRA. FAS was immune precipitated with anti-FAS antibody. The *top panel* indicates the level of the complex between FP-TAMRA and FAS, whereas the *bottom panel (Western blot)* shows the amount of total FAS expression. *C*, effect of Orlistat on cellular fatty acid synthesis was gauged by measuring the incorporation of [$^{14}$C]acetate into fatty acids as described in "Materials and Methods." *D*, the ability of Orlistat to block labeling of the recombinant thioesterase domain of FAS was measured by preincubating the thioesterase with a range of Orlistat and subsequently with the activity probe FP-TAMRA. Samples were run on SDG-PAGE, and signal was visualized as described; *bars*, ±SD.

permeable activity-based probe, FP-BODIPY. Orlistat caused a concentration-dependent inhibition of labeling of FAS by FP-BODIPY (Fig. 3A), indicating that Orlistat inhibits FAS in intact cells. Orlistat had no effect on the abundance of FAS, which was measured from the same treated samples by Western blot. The identity of the labeled FAS was confirmed by immune-precipitating the complex between FP-TAMRA and FAS with an antibody specific for the enzyme (Fig. 3B). The effects of Orlistat on cellular fatty acid synthesis were gauged by measuring the incorporation of [$^{14}$C]acetate into fatty acids. Saturating levels of Orlistat (30 $\mu$M) reduced cellular fatty acid synthesis by ~75% within 30 min (Fig. 3C). Moreover, Orlistat blocked the labeling of the active site serine of the recombinant thioesterase domain of FAS by FP-PEG-TAMRA (Fig. 3D), proving that this enzymatic domain of FAS is a target for Orlistat. Because Orlistat is a tight-binding irreversible inhibitor, we cannot define its precise affinity for FAS. However, the results in Fig. 2D, and similar results obtained on whole FAS in cell lysates (not shown), suggest that the apparent $K_i$ of Orlistat for FAS is near 100 nM. When treating whole cells, however, higher concentrations of the compound were necessary to achieve nearly complete inhibition of the enzyme (Fig. 3A). Orlistat has similar effects on FAS and on fatty acid synthesis in other PCa lines, as well as in colon and breast cancer cell lines (data not shown).

Orlistat induced a pronounced antiproliferative effect in the PC-3 cell line and exhibited a slight effect on the androgen-dependent LNCaP cells. The compound had little effect on the DU-145 cells or the normal PrEC cells in the 48-h time period of the measurement (Fig. 4A). The inhibitory effects of Orlistat on the proliferation of PC-3 cells were reversed by addition of palmitate, the end product of FAS (Fig. 4B). This observation strongly indicates that decreased proliferation results from inhibition of FAS by Orlistat.

We have observed that Orlistat has potent antiproliferative effects on many other tumor cell lines, including cells derived from breast (MDA-MB-435 and MDA-MB-231) and colon (Caco-2 and SW480) cancer. In virtually all of the cases, tumor cells are more sensitive to Orlistat over normal epithelial cells and fibroblasts. Yet, as observed here with PCa cells, variance in the sensitivity of tumor cells to Orlistat is observed (data not shown). The mechanisms underlying these differences are not entirely clear and are currently under investigation. The antitumor activity of anti-FAS compounds has been linked to hormone-dependence (25), PTEN status (26), and Her-2 status (27). Consequently, the sum total of these signaling pathways in any single tumor may ultimately define sensitivity to a FAS blockade.

Orlistat also induced tumor cell apoptosis, but again the sensitivity of the individual tumor cell lines to the compound was somewhat different (Fig. 5A). When cell death was assessed at 24 h by measuring DNA fragmentation (Roche Cell Death ELISA), the PC-3 and LNCaP cells exhibited substantial levels of cell death. At this time point, however, only a modest effect was observed on death of the DU-145 cells. Orlistat was without effect on death of human foreskin fibroblasts or normal PrECs. This finding is consistent with reports that FAS has little, if any, role in apoptosis in normal cells (18). Prolonged exposure (72 h) of each tumor cell line to Orlistat resulted in cleavage of poly(ADP-ribose) polymerase, another marker of apoptosis (Fig. 5B). In this and other apoptosis assays (e.g., staining of annexin on the cell surface), we have yet to observe significant effects of Orlistat (1–25 $\mu$M) on fibroblasts, normal PrECs, or normal mammary epithelial cells (data not shown). As with cell proliferation, the effects of Orlistat on apoptosis were also reversed by the addition of palmitate (Fig. 5C).

The effects of Orlistat on tumor growth *in vivo* were tested in a xenograft model with the PC-3 cells. Tumors were grown to ~100 mm$^3$ in the flank of nude mice, at which time Orlistat was adminis-
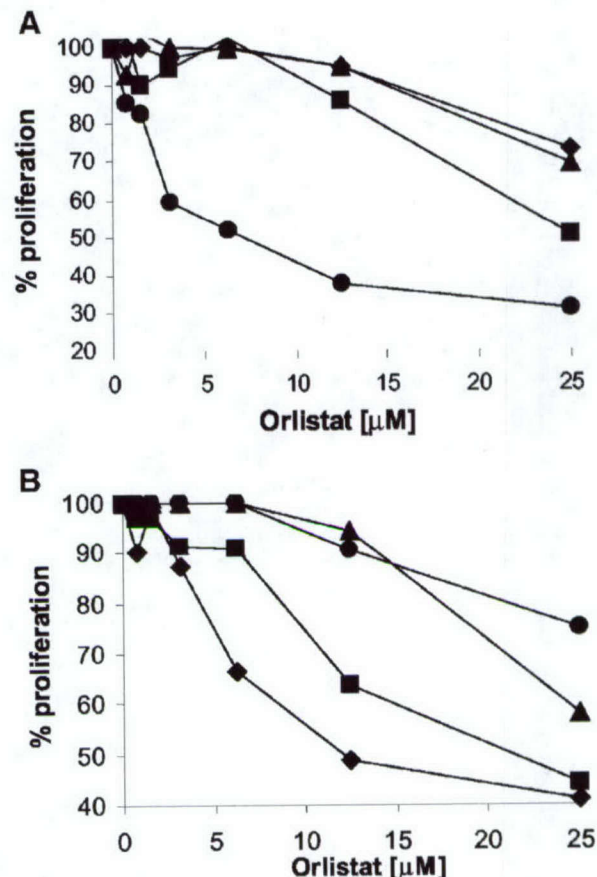


Fig. 4. Orlistat inhibits tumor cell proliferation. A, the effect of Orlistat on proliferation of prostate cancer cells was measured by the incorporation of bromodeoxyuridine (BrdUrd). PC-3 (●), LNCap (■), DU-145 (▲), and PrEC cells (◆) were seeded as subconfluent monolayers into microtiter plates. Cells were exposed to Orlistat for 32 h, then a BrdUrd labeling solution was added to the wells. BrdUrd incorporation was measured according to the manufacturer's protocol (Roche Cell Proliferation ELISA). Proliferation is expressed as a percentage of proliferation in untreated cells. B, the ability of palmitate to rescue cells from Orlistat-induced growth arrest was tested with PC-3 cells. Along with Orlistat, cells were incubated with 0 (◆), 3.25 $\mu$M (■), 7.5 $\mu$M (▲), and 15 $\mu$M (●) palmitate. BrdUrd incorporation was measured according to the manufacturer's protocol (Roche Cell Proliferation ELISA).

tered via i.p. injection (240 mg/kg/day). When compared with vehicle injection, Orlistat prevented the growth of PC-3 tumors (Fig. 6). In five separate experiments such as that shown in Fig. 6, tumor growth was blocked by 63%, 62%, 46%, 41%, and 16%. All of the differences were statistically significant in *t* tests with $Ps < 0.05$. Animals exhibited no outward signs of toxicity, experienced no loss of weight, nor were there any effects of Orlistat (240 mg/kg/day) on hematocrit or WBC levels (data not shown).

A pharmacokinetic analysis of Orlistat (155 mg/kg) administered by i.p. injection showed peak blood levels to be ~10 $\mu$M 2 h after dosing (data not shown). Beyond this time, blood levels of the drug decayed rapidly. Although the cost of a pharmacokinetic analysis at 240 mg/kg was prohibitive, it is unlikely that blood levels reached much beyond 16 $\mu$M, and the half-life is expected to be the same. Altogether then, the peak blood levels at the dose of 240 mg/kg (~16 $\mu$M) are likely to be just above the dose of Orlistat required to affect tumor cells (1–6 $\mu$M). These parameters are generally consistent with the level of growth inhibition that we observe *in vivo*.

Altogether, the findings presented here, and results from our larger survey of a number of tumor and normal cell lines (data not shown), lead to the following conclusions: (a) Orlistat is a novel inhibitor of the thioesterase activity of FAS, and by virtue of this property, Orlistat

can block cellular fatty acid synthesis; (b) Orlistat can interfere with prostate tumor cell proliferation; (c) Orlistat selectively induces apoptosis in prostate tumor cells; (d) tumor cells have various levels of sensitivity to Orlistat; and (e) Orlistat inhibits growth of PC-3 xenograft tumors in vivo.

As with any compound, off-target activity is always a confounding issue. In fact, one reason why we chose to include Orlistat in our study was to determine whether Orlistat had targets other than pancreatic lipase. Nevertheless, the overwhelming body of evidence in this report indicates that the effects of Orlistat on tumor cells are elicited through its inhibition of FAS. Activity-based screening in numerous tumor cells has revealed no other target for Orlistat than FAS. Furthermore, the concentrations of Orlistat that elicit cytostatic and cytotoxic effects are very close to the cellular $IC_{50}$ for the inhibition by Orlistat of FAS. Additional support for this conclusion is drawn from our finding



Fig. 6. Orlistat inhibits growth of PC-3 tumors in vivo. The effect of Orlistat on growth of PC-3 tumors in nude mice was assessed with a staged model. PC-3 cells ($1 \times 10^6$) were injected into the flank of nude mice. Tumors were allowed to grow until they reached a size of ~100 mm$^3$, at which time administration of Orlistat (●) or vehicle (■) was initiated in separate sets of 8 mice. Orlistat was administered at 240 mg/kg/day for a period of 3 weeks. This experiment is representative of four repetitions. The differences in mean tumor volume of the drug versus vehicle groups were statistically significant at every time point with the final measurement at day 28 having a P of 0.02 in a paired t test, assuming unequal variance; bars, ±SD.

that Orlistat directly inhibits the recombinant thioesterase domain of FAS. Finally, palmitate, the end product of FAS, rescues PC-3 cells from the antiproliferative and proapoptotic effects of the compound. This conclusion is also strongly supported by reports in the literature showing that FAS is misregulated in tumors (13) and that c75, an antagonist of the ketoacyl synthase domain, has antitumor activity (8, 20, 22). Finally, recent studies show that silencing of FAS in tumor cells with small interfering RNA induces apoptosis in PCa cells (28). Although we cannot exclude the possibility that Orlistat has some other target, the simplest interpretation of the data presented here is that Orlistat acts by inhibiting FAS.

Orlistat has minimal effects on the normal cells we have tested, suggesting that the compound could have therapeutic index sufficient for antitumor therapy. Orlistat also represents an alternative to cerulenin or c75, which inhibits the ketoacyl synthase domain of the enzyme but also interacts with carnitine palmitoyl transferase (29). In its approved formulation, however, Orlistat is administered orally. Because of its extremely low oral bioavailability, the effects of Orlistat are largely confined to the gastrointestinal tract, where it inactivates pancreatic lipase (24). Therefore, the formulation and route of delivery would have to be changed to treat tumors of the breast, prostate, and so on. One cannot exclude the possibility that the oral formulation of Orlistat could be useful in treating tumors of the gastrointestinal tract, such as colon cancer. We have found Orlistat to block FAS and induce apoptosis in a number of colon cancer lines,[3] so treating patients at high risk for colon cancer in a prophylactic manner could be considered.

The potential for synthesizing more potent or bioavailable variants of Orlistat is high. Orlistat is one of a class of compounds containing a reactive β-lactone. Other compounds in this class include the natural products ebelactones A and B, some inhibitors of HMG CoA synthase (30), and panclicin D, a synthetic inhibitor of pancreatic lipase (31). Given the relatively broad inhibition profile of ebelactones A and B against serine hydrolases (Fig. 2) and the demonstration that synthetic routes are available for creation of variants of Orlistat (30), a more in-depth evaluation of β-lactones as serine hydrolase antagonists and as antitumor agents is warranted.
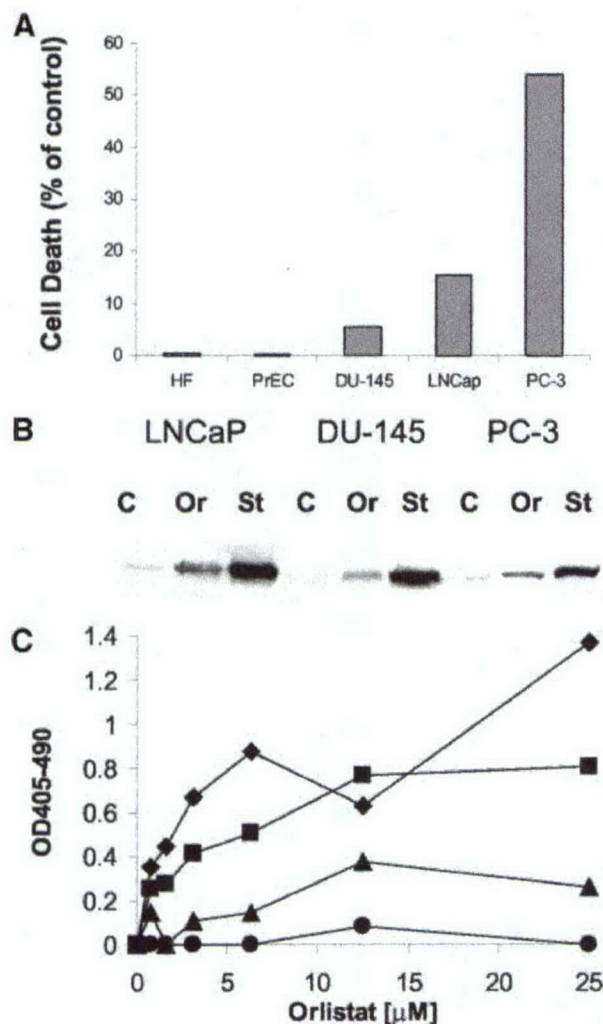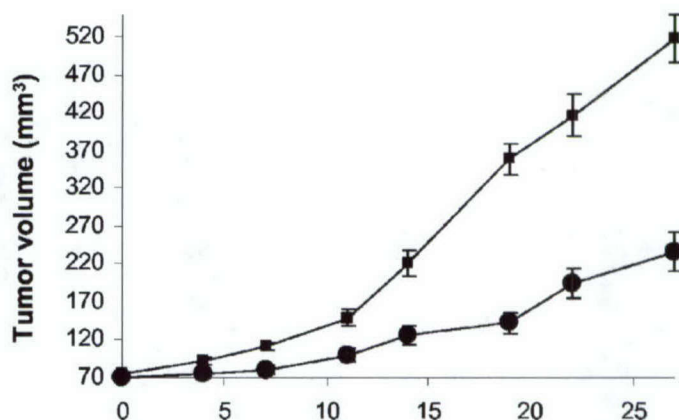


Fig. 5. Orlistat induces tumor cell death. A, PC-3, LNCaP, DU-145, normal prostate epithelial cells (PrECs), and normal foreskin fibroblasts cells were exposed to Orlistat (12.5 μM) for 48 h. Tumor cell death was measured with the Cell Death Detection ELISA (Roche), which measures DNA fragments within immunocaptured nucleosomes. DNA fragmentation was assessed by measuring $A_{405}$-$A_{490}$. The level of cell death is plotted as a percentage of staurosporine induced death. B, LNCaP, DU-145, and PC-3 cells were cultured with Orlistat (12.5 μM) for 72 h, with Staurosporine (St) as a positive control for induction of cell death, or in medium without any stimulus (C). After incubation, cell extracts were generated and subjected to Western analysis using antibodies selective for the cleaved form of poly(ADP-ribose) polymerase. C, PC-3 cells were treated across a concentration range of Orlistat. Concurrently, cells were supplemented with palmitate. Control cells (●) received no palmitate. Test cells were treated with 1.8 μM (▲), 3.75 μM (■), or 7.5 μM (♦) palmitate. After a 48-h treatment, cell death was measured with the Cell Death Detection ELISA (Roche).

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[3] F. Axelrod. Orlistat as an Anti-Tumor Agent for Colorectal Cancer, manuscript in preparation.

Finally, the identification of the ability of Orlistat to inhibit the thioesterase domain of FAS was made possible by the application of activity-based profiling. The analysis we performed involved a screen of only three β-lactones against slightly >35 different serine hydrolases. Even within this small test set, a novel target and indication were identified for an approved drug. It is reasonable to believe that a high throughput version of such a screen could drive decision-making in drug development. Information from such screens could lead to the identification of more selective leads much earlier in development. As in the case of this report, such an analysis might also point toward unanticipated targets and indications for other drugs.

## ACKNOWLEDGMENTS

## REFERENCES

1. Ideker T, Galitski T, Hood L. A new approach to decoding life: systems biology. Ann Rev Genomics Hum Genet 2001;2:343–72.
2. Liu Y, Patricelli MP, Cravatt BF. Activity-based protein profiling: the serine hydrolases. Proc Natl Acad Sci USA 1999;96:14694–9.
3. Greenbaum D, Medzihradszky KF, Burlingame A, Bogyo M. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. Chem Biol 2000;7:569–81.
4. Alo PL, Visca P, Marci A, Mangoni A, Botti C, Di Tondo U. Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. Cancer (Phila) 1996;77:474–82.
5. Alo PL, Visca P, Trombetta G, et al. Fatty acid synthase (FAS) predictive strength in poorly differentiated early breast carcinomas. Tumori 1999;85:35–40.
6. Bull JH, Ellison G, Patel A, et al. Identification of potential diagnostic markers of prostate cancer and prostatic intraepithelial neoplasia using cDNA microarray. Br J Cancer 2001;84:1512–9.
7. Furuya Y, Akimoto S, Yasuda K, Ito H. Apoptosis of androgen-independent prostate cell line induced by inhibition of fatty acid synthesis. Anticancer Res 1997;17:4589–93.
8. Gabrielson EW, Pinn ML, Testa JR, Kuhajda FP. Increased fatty acid synthase is a therapeutic target in mesothelioma. Clin Cancer Res 2001;7:153–7.
9. Gansler TS, Hardman W, III, Hunt DA, Schaffel S, Hennigar RA. Increased expression of fatty acid synthase (OA-519) in ovarian neoplasms predicts shorter survival. Hum Pathol 1997;28:686–92.
10. Kidd D, Liu Y, Cravatt B. Profiling serine hydrolase activities in complex proteomes. Biochemistry 2001;40:4005–15.
11. Landry F, Lombardo CR, Smith JW. a method for application of samples to matrix-assisted laser desorption ionization time-of-flight targets that enhances peptide detection. Anal Biochem 2000;279:1–8.
12. Harvey S, Zhang Y, Landry F, Miller C, Smith JW. Insights into a plasma membrane signature. Physiol Genomics 2001;5:129–36.
13. Kuhajda FP, Jenner K, Wood FD, et al. Fatty acid synthesis: a potential selective target for antineoplastic therapy. Proc Natl Acad Sci USA 1994;91:6379–83.
14. Pizer ES, Chrest FJ, DiGiuseppe JA, Han WF. Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. Cancer Res 1998;58:4611–5.
15. Boven E, Winograd B, Berger DP, et al. Phase II preclinical drug screening in human tumor xenografts: a first European multicenter collaborative study. Cancer Res 1992;52:5940–7.
16. Patricelli MP, Giang DK, Stamp LM, Burbaum JJ. Direct visualizaiton of serine hydrolase activities in complex proteomies using fluorescent active site-directed probes. Proteomics 2001;1:1067–71.
17. Wakil SJ. Fatty acid synthase, a proficient multifunctional enzyme. Biochemistry 1989;28:4523–30.
18. Kuhajda FP. Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. Nutrition 2000;16:202–8.
19. Moche M, Schneider G, Edwards P, Dehesh K, Lindqvist Y. Structure of the complex between the antibiotic cerulenin and its target, β-ketoacyl-acyl carrier protein synthase. J Biol Chem 1999;274:6031–4.
20. Kuhajda FP, Pizer ES, Li JN, Mani NS, Frehywot GL, Townsend CA. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. Proc Natl Acad Sci USA 2000;97:3450–4.
21. Pizer ES, Wood FD, Heine HS, Romantsev FE, Pasternack GR, Kuhajda FP. Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. Cancer Res 1996;56:1189–93.
22. Pizer ES, Pflug BR, Bova GS, Han WF, Udan MS, Nelson JB. Increased fatty acid synthase as a therapeutic target in androgen-independent prostate cancer progression. Prostate 2001;47:102–10.
23. Hadvary P, Sidler W, Meister W, Vetter W, Wolfer H. The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase. J Biol Chem 1991;266:2021–7.
24. McNeely W, Benfield P. Orlistat. Drugs 1998;56:241–249.discussion 250.
25. Swinnen JV, Esquenet M, Goossens K, Heyns W, Verhoeven G. Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. Cancer Res 1997;57:1086–90.
26. Van de Sande T, De Schrijver E, Heyns W, Verhoeven G, Swinnen JV. Role of the phosphatidylinositol 3′-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. Cancer Res 2002;62:642–6.
27. Kumar-Sinha C, Ignatoski KW, Lippman ME, Ethier SP, Chinnaiyan AM. Transcriptome analysis of HER2 reveals a molecular connection to fatty acid synthesis. Cancer Res 2003;63:132–9.
28. De Schrijver E, Brusselmans K, Heyns W, Verhoeven G, Swinnen JV. RNA interference-mediated silencing of the fatty acid synthase gene attenuates growth and induces morphological changes and apoptosis of LNCaP prostate cancer cells. Cancer Res 2003;63:3799–804.
29. Thupari JN, Landree LE, Ronnett GV, Kuhajda FP. C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity.[comment]. Proc Natl Acad Sci USA 2002;99:9498–502.
30. Romo D, Harrison PH, Jenkins SI, et al. Synthesis and inhibitory action on HMG-CoA synthase of racemic and optically active oxetan-2-ones (β-lactones). Bioorg Med Chem 1998;6:1255–72.
31. Yang HW, Zhao CX, Romo D. Studies of the tandem mukaiyama aldollactonization (TMAL) reaction: a concise and highly diastereoselective route to B-lactones applied to the total synthesis of the potent pancreatic lipase inhibitor, (-)-Panclicin D. Tetrahedron 1997;53:16471–88.

# A Fatty Acid Synthase Blockade Induces Tumor Cell-cycle Arrest by Down-regulating Skp2*S

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In eukaryotes, fatty acid synthase (FAS) is the enzyme responsible for synthesis of palmitate, the precursor of long-chain nonessential fatty acids. FAS is up-regulated in a wide range of cancers and has been suggested as a relevant drug target. Here, two independent approaches are taken toward knocking down FAS and then probing its connection to tumor cell proliferation. In one approach, Orlistat, a drug approved for treating obesity, is used as a potent inhibitor of the thioesterase function of FAS. In a separate strategy, the expression of FAS is suppressed by targeted knock-down with small interfering RNA. In both circumstances, the ablation of FAS activity causes a dramatic down-regulation of Skp2, a component of the E3 ubiquitin ligase that controls the turnover of p27$^{Kip1}$. These effects ultimately tie into the retinoblastoma protein pathway and lead to a cell-cycle arrest at the $G_1/S$ boundary. Altogether, the findings of the study reveal unappreciated links between fatty acid synthase and ubiquitin-dependent proteolysis of cell-cycle regulatory proteins.

Breast cancer is the second-leading cause of cancer death and morbidity for women in the United States (1, 2). Although advances in early detection and treatment have led to a decline in mortality, the survival rate for patients with advanced-stage breast cancer is still low (1). Consequently, there is still a great need to identify and validate new molecular targets for antitumor therapy. This study focuses on mammary carcinoma, where fatty acid synthase (FAS)[1] has attracted considerable attention as a potential drug target. Much of the interest in FAS stems from the fact that the enzyme is up-regulated in about 50% of breast cancers and is an indicator of poor prognosis (3–7).

FAS is the enzyme responsible for cellular synthesis of palmitate, the precursor of long-chain nonessential fatty acids (8–11). FAS, which contains seven separate enzymatic pockets, is situated as a head-to-tail dimer with the ketoacyl synthase and malonyl/acetyl transferase domains of one monomer working together with the dehydratase, enoyl reductase, ketoacyl reductase, acyl carrier protein, and thioesterase domains on the adjacent monomer (8–11). These enzymatic domains act sequentially to condense acetyl-CoA with malonyl-CoA to form a four-carbon intermediate. Six additional turns of the cycle of the enzyme convert this intermediate to palmitate, which is then liberated from FAS by the action of the thioesterase domain (12).

Because FAS functions as a head-to-tail dimer, targeted inhibition of one of the enzymatic domains of FAS can ablate the activity of one or both FAS subunits (10, 11). Cerulenin, a natural product, is an antagonist of the ketoacyl synthase domain (the condensing enzyme) of FAS and functions by covalently modifying the active site cysteine, resulting in dead-end inhibition (13). c75, a synthetic analog of cerulenin, also targets the condensing enzyme and inhibits fatty acid synthesis (14). The inhibition of FAS by either cerulenin or c75 can suppress tumor cell proliferation and, in some cases, can induce tumor cell apoptosis (14–20). These observations support the contention that FAS is a relevant drug target in oncology. However, both cerulenin and c75 are now known to have other molecular targets (21–23), so searches for additional antagonists of FAS with better selectivity and distinct mechanisms of action are certainly warranted.

We recently reported (24) that Orlistat, a drug approved for treating obesity, is a rather potent and selective inhibitor of FAS in prostate carcinoma cells. The drug elicits its effects by inhibiting the thioesterase domain of FAS, which is responsible for releasing palmitate from the acyl carrier protein of the enzyme. By virtue of this activity, Orlistat slowed the growth of xenograft tumors of PC-3 prostate carcinoma cells in mice (24). The objective of the present study was two-fold. First, we sought to use an independent strategy to confirm that the antiproliferative effects of Orlistat result from inhibition of FAS. Second, we sought to elucidate the mechanism by which a FAS blockade interferes with tumor cell proliferation. Both Orlistat and small interfering RNA (siRNA)-targeting FAS cause a dramatic down-regulation of Skp2, a component of an E3 ubiquitin ligase that tags p27$^{Kip1}$ for degradation by the proteasome. These findings mechanistically connect two biochemical pathways being explored as drug targets in cancer, fatty acid biosynthesis, and ubiquitin-dependent proteolysis.

## MATERIALS AND METHODS

*Cell Lines*—The MDA-MB-435 cell line was obtained from Janet Price at the University of Texas Southwestern Medical Center. MDA-MB-231 and MCF7 were purchased from American Type Culture Collection (Manassas, VA). Tumor cells were maintained in minimal Eagle's media, Earle's salts (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (Irvine Scientific), 2 mM L-glutamine (Invitrogen), minimal Eagle's media vitamins (Invitrogen), nonessential amino acids (Irvine Scientific) and antibiotics (Omega Scientific, Inc., Tarzana, CA).

[1] The abbreviations used are: FAS, fatty acid synthase; siRNA, small interfering RNA; PBS, phosphate-buffered saline; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; FP-PEG-TAMRA, fluorophosphonate-poly-ethyleneglycol-tetramethyl rhodamine.

*Profiling Serine Hydrolase Activity in Mammary Epithelial Cells*—Cultured cells were washed with ice-cold phosphate-buffered saline (PBS), harvested with a cell scraper, and collected by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl (pH 8.0) and lysed by sonication as described previously (25, 26). The soluble and insoluble cell fractions were separated by ultracentrifugation at 64,000 rpm for 1 h at 4 °C. Protein concentrations were determined by using the BCA assay kit from Pierce. The resulting extracts were diluted in lysis buffer to yield a 1 mg/ml final protein concentration.
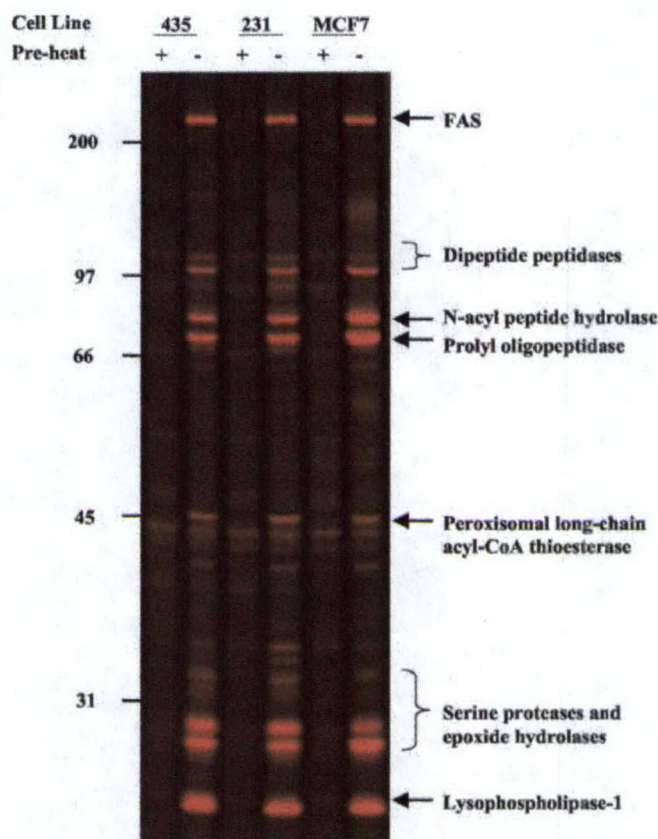
The fluorophosphonate probes FP-PEG-TAMRA (fluorophosphonate-poly-ethyleneglycol-tetramethyl rhodamine), FP-PEG-BODIPY, and FP-PEG-biotin were synthesized and generously provided by Activx Biosciences (La Jolla, CA). Serine hydrolase activity was examined by incubating the soluble cell fractions (40 $\mu$l) with FP-PEG-TAMRA (2 $\mu$M) for 1 h at room temperature. Nonspecific labeling was monitored by denaturing samples for 10 min at 100 °C prior to labeling with FP-PEG-TAMRA. The reaction was terminated by the addition of 2× Laemmli sample buffer, boiled for 5 min, and resolved by SDS-10% PAGE. Fluorescent-labeled hydrolases were visualized at 605 nm using a Hitachi flatbed scanner and quantitated with Image Analysis (MiraiBio, Alameda, CA).

*Purification and Identification of Serine Hydrolases by Avidin-biotin Affinity Chromatography and Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry*—Serine hydrolases were identified using the FP-PEG-biotin probe (26). Briefly, cell lysates were preabsorbed with avidin-agarose to reduce nonspecific binding of proteins during purification. Lysates were labeled with FP-PEG-biotin (5 $\mu$M) at room temperature for 1 h, after which proteins were separated from unincorporated FP-PEG-biotin by gel filtration on Nap 25 columns. After the addition of 0.5% SDS, the eluate was boiled for 10 min to denature proteins. Samples were diluted with 50 mM Tris (pH 7.5) and 150 mM NaCl and absorbed with avidin-agarose for 1 h at room temperature. Avidin-agarose beads were pelleted by centrifugation and washed 8× with 50 mM Tris (pH 7.5), 150 mM NaCl, and 1% Tween 20. Labeled proteins were eluted with 2× sample buffer, resolved by SDS-10% PAGE, and detected by silver staining. Specific bands were extracted and subjected to in-gel trypsin digestion and peptide mass fingerprinting with MALDI-TOF using methods described previously (27, 28).

*Inhibition of Serine Hydrolase Activity with Orlistat*—Orlistat was extracted from Xenical™ capsules (Roche Applied Science) by solubilizing each pill in 1 ml of ethanol. Insoluble product was removed by centrifugation (14,000 rpm for 5 min). The supernatant yielded a solution of Orlistat (250 mM), which was aliquoted and stored at −80 °C. Soluble cell extracts (40 $\mu$l) were incubated with Orlistat (0–1 $\mu$M) for 20 min prior to FP-PEG-TAMRA addition. Lysates were labeled with FP-PEG-TAMRA, and reactions were terminated and processed as described above. The final concentration of Me$_2$SO or ethanol in each reaction was 10%.

*Gene Silencing Using siRNA*—FAS siRNA sequences corresponding to 5′-CAA CTA CGG CTT TGC CAA T (nucleotides 6213–6231), 5′-GCA ACT CAC GCT CCG GAA A (nucleotides 6657–6675), 5′-GCC CTG AGC TGG ACT ACT T (nucleotides 6146–6164), and 5′-GGT ATG CGA CGG GAA AGT A (nucleotides 7515–7533) were custom designed and pooled together by Dharmacon (Lafayette, CO). MDA-MB-435 cells were plated at 3.125 × 10$^4$/cm$^2$ in 6-cm plates for 24 h prior to transfection with 100 nM FAS, Skp2 (Dharmacon Smartpool M-003324–01) or scrambled control (Dharmacon D-001206–13) siRNA in Opti-MEM medium (Invitrogen) using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were placed into normal culture medium 6 h post-transfection and grown for an additional 42 h.

*Fatty Acid Biosynthesis*—The incorporation of [$^{14}$C]malonyl CoA into cellular fatty acids was measured according to published methods (15). Briefly, MDA-MB-435 cells were harvested with a cell scraper and centrifuged at 2,000 rpm for 5 min and then frozen at −80 °C. Cell pellets were hypotonically lysed in 20 mM Tris (pH 7.5), 1 mM dithiothreitol, and 1 mM EDTA, and insoluble material was removed by centrifugation (14,000 rpm) for 15 min at 4 °C. Lysates (380 $\mu$g) were exposed to Orlistat (0.1–10 $\mu$M) or vehicle at 25 °C for 1 h. Lysates in 80-$\mu$l volumes were added to 520 $\mu$l of solution containing 581 $\mu$M NADPH, 193 $\mu$M acetyl CoA, and 116 mM KCl (pH 6.6). Reactions were mixed with 0.4 $\mu$Ci [2-$^{14}$C]malonyl CoA (Amersham Biosciences) for 25 min at 37 °C. Cold malonyl CoA (208 $\mu$M) was added to reaction mixtures, which were incubated for an additional 15 min at 37 °C. Reactions were terminated by the addition of chloroform:methanol (1:1). The chloroform extracts were dried under N$_2$ and extracted with water-saturated butanol. The butanol extract was evaporated under N$_2$, and



| Cell Line | 435 | 231 | MCF7 |
|---|---|---|---|
| Pre-heat | + − | + − | + − |

FAS

Dipeptide peptidases

N-acyl peptide hydrolase
Prolyl oligopeptidase

Peroxisomal long-chain acyl-CoA thioesterase

Serine proteases and epoxide hydrolases

Lysophospholipase-1

FIG. 1. **Serine hydrolase profile of mammary carcinoma cells.** Cell lysates were generated from three breast cancer cell lines (MDA-MB-435, MDA-MB-231, and MCF7). Lysates were labeled with FP-PEG-TAMRA to tag active serine hydrolases (*lanes* marked with −). Nonspecific labeling with the probe was measured in samples that were boiled prior to the addition of FP-PEG-TAMRA (*lanes* marked with +).

labeled fatty acids were quantified by scintillation counting. The identity of the labeled fatty acid was verified by comparison to a palmitate standard on thin layer chromatography. Briefly, lipid extracts from Orlistat and vehicle-treated lysates of MDA-MB-435 cells were resuspended in 40 $\mu$l of chloroform, spotted on silica gel (EM Science), and chromatographed in hexane/diethyl ether/acetic acid (45:5:1). Tritiated palmitate (PerkinElmer Life Sciences) and cold palmitate (Sigma) were used as standards. Chromatographed lipids were detected by exposing the plate in iodine vapor and on Biomax film (Kodak).
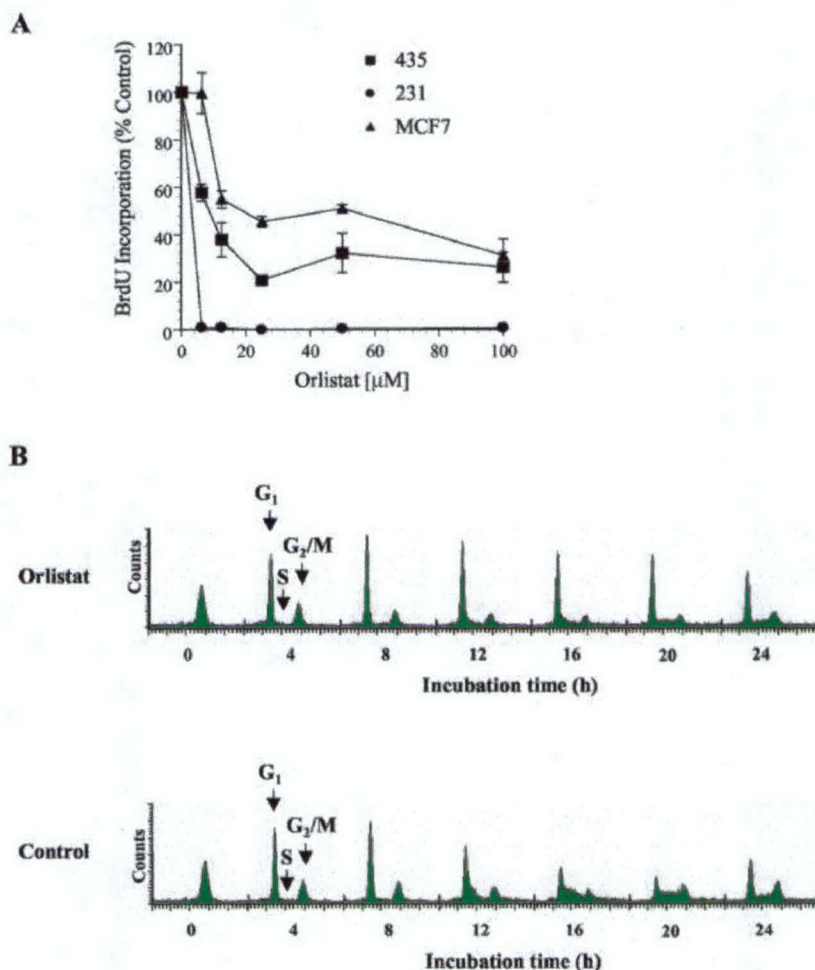
*Cell Proliferation Assays*—Cells were plated at 6.25 × 10$^4$/cm$^2$ in 96-well plates for 24 h. Cells were washed with PBS and incubated in serum-free RPMI 1640 medium for 24 h prior to the addition of Orlistat (0–100 $\mu$M). Cell proliferation was measured 72 h later using the cell proliferation BrdU ELISA kit (Roche) according to the manufacturer's directions.

*Cell Synchronization and Cell-cycle Analysis*—Tumor cells were plated at 6.25 × 10$^4$/cm$^2$ in 6-well plates for 24 h, washed with PBS, and serum-starved for an additional 24 h. M phase synchronization was achieved by treating cells with 100 nM nocodazole (Sigma) for 16 h. Synchronized cells were treated with Orlistat (0–50 $\mu$M) immediately after release from the block and harvested at various times over 24 h. Cells were collected by trypsinization, rinsed in cold PBS, fixed in 70% ethanol, and stored at −20 °C. Cellular DNA was stained by the addition of PBS containing 200 units/ml of RNase (Roche) and 18 $\mu$g/ml of propidium iodide (Molecular Probes, Eugene, OR). Fluorescence was monitored on 15,000 cells per sample using a BD FACSort tabletop cytometer (BD Biosciences). Data were analyzed with Modfit LT software (Verity Software House, Topsham, ME).

*Western Blot Analysis*—Cells were synchronized with nocodazole, treated with Orlistat (0–50 $\mu$M), harvested by trypsinization, and rinsed with PBS. Pellets were lysed in 2× SDS sample buffer, passed 20 times through an 18-gauge needle, boiled for 5 min, and stored at −80 °C. Insoluble material was removed by centrifugation (14,000 rpm) for 10 min at 4 °C, and protein was separated by SDS-PAGE. After electrophoresis, protein was transferred onto nitrocellulose and probed

*Fatty Acid Synthase and Skp2**

**A**



**B**



FIG. 2. **Orlistat inhibits tumor cell proliferation by blocking $G_1/S$ progression.** *A*, the effect of Orlistat on cell proliferation was monitored by measuring bromodeoxyuridine incorporation into cellular DNA. MDA-MB-435, MDA-MB-231, and MCF7 cells were exposed to Orlistat (0–100 μM) for 72 h and monitored for DNA synthesis during the final 24 h of treatment. Cell growth is normalized to untreated controls and expressed as a percentage of proliferation. Values are means + S.E. of four replicates per treatment. *B*, MDA-MB-435 cells were synchronized in the M phase using nocodazole. After nocodazole removal and refeeding with fresh media at time 0, cells were exposed to 50 μM Orlistat (*upper panel*) or vehicle control (*lower panel*) and analyzed at various time points during a 24-h period. The distribution of cells in different phases of the cell cycle was monitored as a function of time using flow cytometry. The panels show representative flow cytometry profiles obtained from two experiments.

overnight at 4 °C with anti-FAS (BD Transduction Laboratories, San Jose, CA), anti-p27 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-tubulin (Santa Cruz Biotechnology), anti-Rb (Santa Cruz Biotechnology), anti-phospho-Rb (Ser-780; Cell Signaling Technology, Beverly, MA), anti-phospho-Rb (Ser-795; Cell Signaling Technology), anti-phospho-Rb (Ser-907/811; Cell Signaling Technology), and anti-p45 Skp2 (Zymed Laboratories, Inc., South San Francisco, CA). Immunoreactivity was detected by using anti-mouse or anti-rabbit IgG-conjugated peroxidase and visualized by enhanced chemiluminescence.

### RESULTS

*Serine Hydrolase Profile of Normal and Neoplastic Mammary Epithelial Cells*—Activity-based protein profiling was used to visualize the profile of active serine hydrolases present in mammary carcinoma cells. We used an activity-based probe of serine hydrolases consisting of a fluorophosphonate moiety linked to tetramethyl rhodamine (FP-PEG-TAMRA). The fluorophosphonate warhead tags the active site serine of serine hydrolases, forming an adduct that is stable to SDS gel electrophoresis (29). Activity profiles of three human breast cancer cell lines, MCF7, MDA-MB-231, and MDA-MB-435, were compared (Fig. 1). In each case, more than 20 active serine hydrolases were detected as fluorescent bands on the SDS gel. Preheating the sample to denature the enzymes in the lysate eliminated reaction with the FP-PEG-TAMRA probe. The identity of many of these enzymes was determined with mass spectrometry. They included, among others, dipeptidyl peptidases 7 and 9, prolyloligopeptidase, lysophospholipase-1, and FAS.

*Orlistat Suppresses Tumor Cell Proliferation by Interfering with $G_1/S$ Progression*—Our previous work (24) showed that
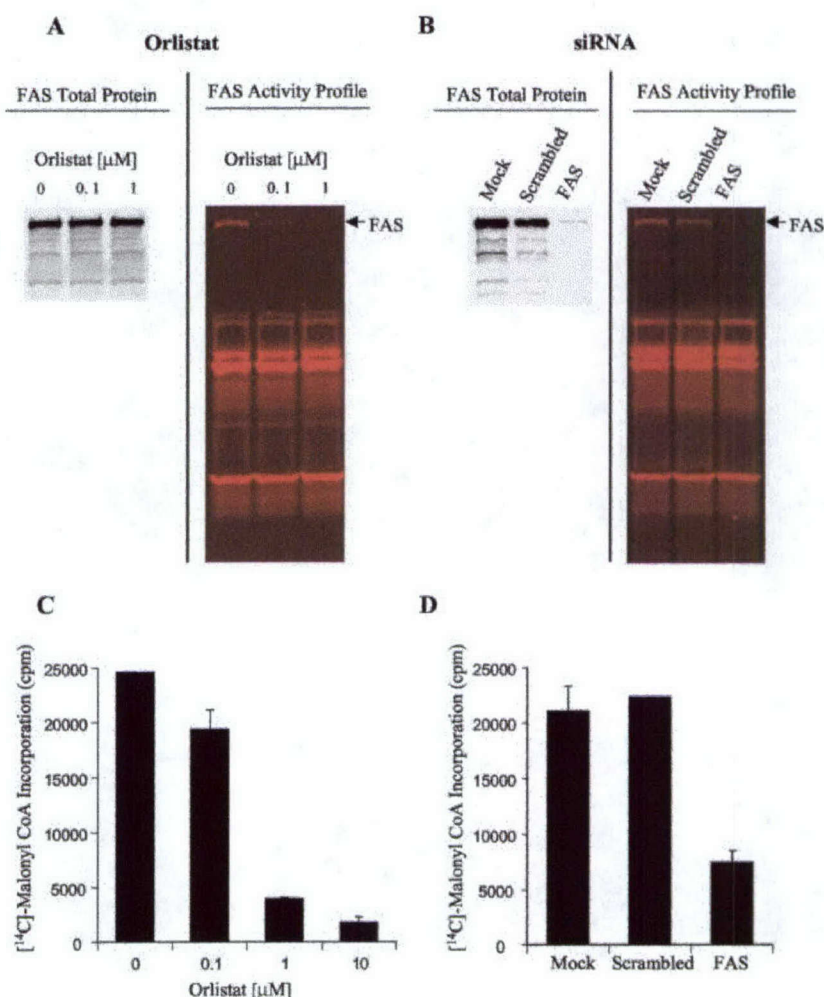
Orlistat inhibits FAS in prostate carcinoma cells and that such inhibition slows their growth *in vivo*. Given the presence of FAS in mammary carcinoma cells (Fig. 1), we conducted studies to determine whether Orlistat would inhibit their proliferation. Studies were conducted to determine whether Orlistat interferes with the proliferation of mammary carcinoma cells. Cells were incubated with Orlistat for 72 h, and DNA synthesis was measured by incorporation of bromodeoxyuridine. Orlistat inhibited proliferation of the MCF7, MDA-MB-231, and MDA-MB-435 cell lines (Fig. 2A). Slight differences were observed in the response of each cell line to Orlistat. Although proliferation of the MDA-MB-231 cells was completely inhibited by Orlistat, proliferation of the MDA-MB-435 cells was knocked down by 70–80%, and proliferation of the MCF7 cells was suppressed by ~50%.

To determine the effect of Orlistat on cell-cycle progression, we used synchronized cultures of MDA-MB-435 cells. Cells were synchronized in the M phase using a nocodazole block. After release of the block, cells were exposed to a saturating concentration of Orlistat and analyzed for the distribution of cells in the $G_1$ and S phases every 4 h by flow cytometry. Orlistat dramatically slowed the entry of the cells into S phase compared with untreated cells (Fig. 2B). The amplitude of the $G_1$ peak in Orlistat-treated cells declines slowly after a period of several hours. This decline in the $G_1$ population results partially from apoptosis[2] and also partially because the blockade is leaky. Similar results were obtained when cells were

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[2] L. M. Knowles and J. W. Smith, unpublished observation.

FIG. 3. **Orlistat and siRNA-targeting FAS block fatty acid synthesis.** *A*, the effect of Orlistat upon serine hydrolase activity was measured by incubating MDA-MB-435 cell lysates with the drug for 20 min. The level of FAS protein was quantified by Western blotting (*left panel*), and the profile of active serine hydrolases was probed with the activity-based probe FP-PEG-TAMRA (*right panel*). *B*, the effect of siRNA-targeting FAS was gauged by transfecting MDA-MB-435 cells with FAS siRNA, scrambled siRNA, or only LipofectAMINE reagent. The level of FAS protein was measured by Western blotting (*left panel*), and the level of active FAS thioesterase was gauged with the activity-based probe FP-PEG-TAMRA (*right panel*). *C*, the effect of Orlistat on the synthesis of palmitate was measured in lysates of MDA-MB-435 cells incubated with 0.1–10 $\mu$M Orlistat for 1 h. Subsequently, the lysate was incubated with [$^{14}$C]malonyl CoA, and labeled fatty acids were extracted and quantified by scintillation counting. Values are means + S.E. of three replicates per treatment. *D*, the effect of siRNA-targeting FAS on palmitate synthesis was measured 48 h after transfection using the approaches described for *B*.

synchronized at the $G_1$/S border using thymidine, released from the block, and allowed to progress through the next cell cycle (data not shown).

*Orlistat and Anti-FAS siRNA Block Fatty Acid Biosynthesis*—We sought an independent means of inhibiting FAS to solidify the role of this enzyme in regulating tumor cell proliferation. Therefore, we compared Orlistat and siRNA-targeting FAS for the ability to knock down the activity of the enzyme in MDA-MB-435 mammary carcinoma cells (Fig. 3). As expected, Orlistat was without effect upon the level of FAS (Fig. 3*A*, *left panel*) but did ablate the activity of the thioesterase domain as indicated with the activity-based probe FP-PEG-TAMRA (Fig. 3*A*, *right panel*). The siRNA-targeting FAS reduced the level of FAS protein (Fig. 3*B*, *left panel*) and thereby reduced the labeling of the enzyme with the activity-based probe (Fig. 3*B*, *right panel*). Both antagonists also blocked the synthesis of palmitate, the end product of FAS, as indicated by reductions to the incorporation of [$^{14}$C]malonyl CoA into fatty acids (Fig. 3, *C* and *D*).

*An FAS Blockade Alters the Key Regulatory Steps in the Retinoblastoma Protein Pathway*—We examined the effects of a FAS blockade on key regulatory steps in the retinoblastoma protein (Rb) protein pathway, a primary regulator of the $G_1$/S transition (30). This analysis included measures of (*i*) the phosphorylation status of Rb, a parameter that governs the interaction of this protein with E2F-1 and subsequent entry into S phase (30); (*ii*) p27$^{\text{Kip1}}$, which negatively regulates cyclin-dependent kinase activity (31); and (*iii*) Skp2, a protein component of the E3 ubiquitin ligase that regulates degradation of

p27$^{\text{Kip1}}$ (32). The effect of a concentration range of Orlistat on each of these parameters was measured by Western blotting (Fig. 4*A*). Orlistat reduced phosphorylation of the Rb protein, up-regulated p27$^{\text{Kip1}}$, and down-regulated Skp2. These effects were evident at levels of the drug consistent with the cellular IC$_{50}$ of Orlistat for inhibition of the FAS thioesterase ($\sim$1–3 $\mu$M). To independently verify that the effects of Orlistat could be attributed to its ability to block FAS, similar experiments were conducted with siRNA-targeting FAS (Fig. 4*B*). Like Orlistat, the siRNA-targeting FAS decreased the phosphorylation of Rb, increased the level of p27$^{\text{Kip1}}$, and reduced the level of Skp2. An siRNA-targeting Skp2 had identical effects upon its downstream target, p27$^{\text{Kip1}}$, and upon the phosphorylation status of the Rb protein. Together, these findings provide strong support for the idea that a FAS blockade acts upon the Rb pathway via regulation of Skp2 and also indicate that a FAS blockade is likely to have effects similar to an Skp2 blockade.

## DISCUSSION

Results from this study reinforce the idea that FAS is a relevant drug target in oncology and further support the notion that FAS is the relevant target for Orlistat in tumor cells. The inhibitory effects of Orlistat on FAS are rather unexpected, because the drug has been studied for more than 15 years with no mention of the effects upon fatty acid synthesis. The effects of the drug on FAS are likely to have been overlooked because Orlistat is administered orally but is not significantly absorbed into the bloodstream. The drug acts in preventing absorption of dietary fat by inhibiting pancreatic lipase (another serine hy-
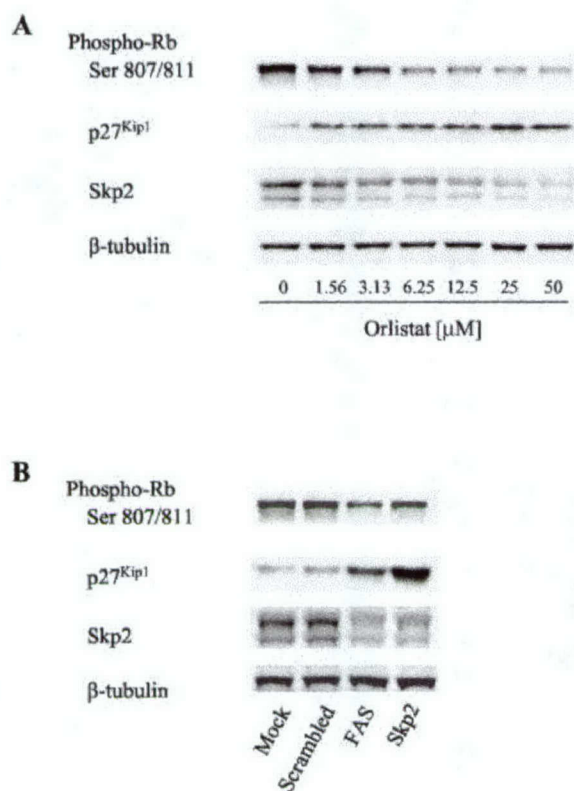
*Fatty Acid Synthase and Skp2*

**A**

Phospho-Rb
Ser 807/811

p27^Kip1

Skp2

β-tubulin

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 0 | 1.56 | 3.13 | 6.25 | 12.5 | 25 | 50 |

Orlistat [μM]

**B**

Phospho-Rb
Ser 807/811

p27^Kip1

Skp2

β-tubulin

Mock  Scrambled  FAS  Skp2

FIG. 4. **Inhibition of FAS leads to down-regulation of Skp2.** *A*, the effect of Orlistat on Rb phosphorylation and on the levels of p27^Kip1 and Skp2 was measured in nocodazole-synchronized MDA-MB-435 cells. Cells were exposed to a range of Orlistat for 20 h, lysed, and subjected to analysis by Western blotting. *B*, the effect of siRNA-targeting FAS or Skp2 on Rb phosphorylation, and the levels of p27^Kip1 were measured in MDA-MB-435 cells. Following a 48-h incubation after transfection with siRNA, cells were lysed and samples were analyzed by Western blotting.

drolase) in the digestive tract (33, 34). The results of the present study indicate that Orlistat and other β-lactones should be considered to be a promising class of thioesterase antagonists that could be exploited for antitumor therapy.

The evidence supporting the conclusion that the antiproliferative effects of Orlistat are mediated by inhibition of FAS is as follows: first, our activity-based protein-profiling experiments indicate that FAS is the only serine hydrolase target for Orlistat in the breast cancer cell lines. We have noted three other bands with $M_r \sim 90$–150 kDa that are also inhibited by Orlistat, but these seem to be breakdown products of FAS because they can be immunoprecipitated with anti-FAS antibody and because they are knocked down with siRNA-targeting FAS (see Fig. 3B). Second, Orlistat blocks the incorporation of [$^{14}$C]malonyl CoA into palmitate, a biosynthetic reaction mediated by FAS. Third, the effects of Orlistat on tumor cell proliferation and regulation of Rb, p27^Kip1, and Skp2 all occur at concentrations of the drug that approximate its cellular IC$_{50}$ for FAS (between 1 and 3 μM). Fourth, our prior work shows that Orlistat directly inhibits the activity of the recombinant thioesterase of FAS (24). Fifth, the effects of Orlistat on tumor cell proliferation and on regulation of Rb and p27^Kip1 are mimicked by siRNA-targeting FAS.

The present study also provides a new insight into the mechanisms underlying the connection between FAS and tumor cell proliferation. Orlistat arrests the cell cycle at the G$_1$/S transition. This effect was noted in all breast cancer cells we tested, along with tumor cells derived from the prostate (not shown). These findings suggest that the block in G$_1$/S progression is a common mechanism mediating the antiproliferative effects of

Orlistat. Substantial decreases in DNA synthesis have likewise been noted in response to cerulenin and c75, other antagonists of FAS (19).

The G$_1$/S cell-cycle arrest elicited by a FAS blockade is mediated through the Rb pathway. Cells treated with either Orlistat or siRNA-targeting FAS show decreased phosphorylation of Rb and increased levels of the complex between Rb and E2F-1 (data not shown). The increased association of these two proteins prevents the transcriptional activity of E2F-1 essential for entry into S phase. We have traced the effects of a FAS blockade upstream of Rb and found such a blockade to alter the levels of p27^Kip1 and Skp2. p27^Kip1 acts as a negative regulator of the cyclin-dependent kinases that ultimately phosphorylate Rb and, therefore, p27^Kip1 acts as a negative regulator of G$_1$/S transition (35). Inhibition of FAS led to increases in p27^Kip1 protein levels without affecting its transcription (data not shown), indicating a stabilization of p27^Kip1. Interestingly, inhibition of FAS also substantially reduces the levels of Skp2, an F-box protein essential for proteasome degradation of p27^Kip1. Like siRNA-targeting FAS, an siRNA-targeting Skp2 also increased p27^Kip1 levels and blocked phosphorylation of Rb. Consequently, we conclude that inhibition of FAS acts upstream of the proteasome to control p27^Kip1 levels and ultimately blocks cell-cycle progression. The mechanistic connections between the FAS blockade by Orlistat and reductions in Skp2 are the subject of current investigations.

The observations in this report are different from prior work in which cerulenin and c75 were employed as FAS inhibitors. These compounds block both G$_1$/S and G$_2$/M progression (16, 19, 36). We found no evidence that FAS blockade with Orlistat affected the G$_2$/M transition point. There are two potential explanations for this difference in effect. One possibility is that inhibition of the different enzymatic pockets of FAS elicits distinct downstream effects. Although we cannot definitively exclude this possibility, it is difficult to envision how antagonists of distinct enzymatic pockets, each leading to inhibition of product formation, could elicit different effects. Another possibility is that cerulenin and c75 bind to additional molecular targets that account for the effects upon G$_2$/M. In this regard, we have begun to analyze clonal variants of MDA-MB-435 cells resistant to Orlistat. We have found these cells to retain sensitivity to cerulenin (data not shown), a finding that lends support to the idea that these two compounds have different mechanisms of action.

It is now clear that several avenues for antitumor therapy converge at the G$_1$/S transition. Recent work indicates that Skp2 is one of the important G$_1$/S regulatory points because it is necessary for ubiquitin-dependent degradation of p27^Kip1 (32). Consequently, Skp2 is a positive regulator of cell-cycle progression. In fact, Skp2 has even been suggested as a potential drug target (37), an idea that is significantly strengthened by the recent observation that Skp2 levels are associated with reduced survival in prostate cancer (38). The present study shows that a FAS blockade ultimately decreases Skp2 levels. Therefore, FAS represents an upstream leverage point for targeting Skp2. Identifying and understanding the mechanism and molecular players that make this connection is an important direction for future investigation.

REFERENCES

1. Weir, H. K., Thun, M. J., Hankey, B. F., Ries, L. A., Howe, H. L., Wingo, P. A., Jemal, A., Ward, E., Anderson, R. N., and Edwards, B. K. (2003) *J. Natl. Cancer Inst.* **95,** 1276–1299
2. McDonald, J. A., Quade, B. J., Broekelmann, T. J., LaChance, R. R., Forsman, K., Hasegawa, E., and Akiyama, S. (1987) *J. Biol. Chem.* **262,** 2957–2967
3. Alo, P. L., Visca, P., Marci, A., Mangoni, A., Botti, C., and Di Tondo, U. (1996) *Cancer* **77,** 474–482
4. Alo, P. L., Visca, P., Trombetta, G., Mangoni, A., Lenti, L., Monaco, S., Botti, C., Serpieri, D. E., and Di Tondo, U. (1999) *Tumori.* **85,** 35–40

5. Wang, Y., Kuhajda, F. P., Li, J. N., Pizer, E. S., Han, W. F., Sokoll, L. J., and Chan, D. W. (2001) *Cancer Lett.* **167,** 99–104
6. Milgraum, L. Z., Witters, L. A., Pasternack, G. R., and Kuhajda, F. P. (1997) *Clin. Cancer Res.* **3,** 2115–2120
7. Alo, P. L., Visca, P., Botti, C., Galati, G. M., Sebastiani, V., Andreano, T., Di Tondo, U., and Pizer, E. S. (2001) *Am. J. Clin. Pathol.* **116,** 129–134
8. Tsukamoto, Y., Wong, H., Mattick, J. S., and Wakil, S. J. (1983) *J. Biol. Chem.* **258,** 15312–15322
9. Chirala, S. S., Jayakumar, A., Gu, Z. W., and Wakil, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3104–3108
10. Rangan, V. S., Joshi, A. K., and Smith, S. (2001) *Biochemistry* **40,** 10792–10799
11. Joshi, A. K., Witkowski, A., and Smith, S. (1997) *Biochemistry* **36,** 2316–2322
12. Mattick, J. S., Nickless, J., Mizugaki, M., Yang, C. Y., Uchiyama, S., and Wakil, S. J. (1983) *J. Biol. Chem.* **258,** 15300–15304
13. Moche, M., Schneider, G., Edwards, P., Dehesh, K., and Lindqvist, Y. (1999) *J. Biol. Chem.* **274,** 6031–6034
14. Kuhajda, F. P., Pizer, E. S., Li, J. N., Mani, N. S., Frehywot, G. L., and Townsend, C. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3450–3454
15. Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., and Pasternack, G. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6379–6383
16. Furuya, Y., Akimoto, S., Yasuda, K., and Ito, H. (1997) *Anticancer Res.* **17,** 4589–4593
17. Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E., and Kuhajda, F. P. (1996) *Cancer Res.* **56,** 2745–2747
18. Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A., and Han, W. F. (1998) *Cancer Res.* **58,** 4611–4615
19. Li, J. N., Gorospe, M., Chrest, F. J., Kumaravel, T. S., Evans, M. K., Han, W. F., and Pizer, E. S. (2001) *Cancer Res.* **61,** 1493–1499
20. Heiligtag, S. J., Bredehorst, R., and David, K. A. (2002) *Cell Death Differ.* **9,** 1017–1025
21. Thupari, J. N., Landree, L. E., Ronnett, G. V., and Kuhajda, F. P. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 9498–9502
22. Lawrence, D. S., Zilfou, J. T., and Smith, C. D. (1999) *J. Med. Chem.* **42,** 4932–4941
23. De Vos, M. L., Lawrence, D. S., and Smith, C. D. (2001) *Biochem. Pharmacol.* **62,** 985–995
24. Kridel, S. J., Axelrod, F., Rozenkrantz, N., and Smith, J. W. (2004) *Cancer Res.* **64,** 2070–2075
25. Liu, Y., Patricelli, M. P., and Cravatt, B. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14694–14699
26. Kidd, D., Liu, Y., and Cravatt, B. (2001) *Biochemistry* **40,** 4005–4015
27. Landry, F., Lombardo, C. R., and Smith, J. W. (2000) *Anal. Biochem.* **279,** 1–8
28. Harvey, S., Zhang, Y., Landry, F., Miller, C., and Smith, J. W. (2001) *Physiol. Genomics* **5,** 129–136
29. Patricelli, M. P., Giang, D. K., Stamp, L. M., and Burbaum, J. J. (2001) *Proteomics* **1,** 1067–1071
30. Brown, V. D., Phillips, R. A., and Gallie, B. L. (1999) *Mol. Cell. Biol.* **19,** 3246–3256
31. Loden, M., Nielsen, N. H., Roos, G., Emdin, S. O., and Landberg, G. (1999) *Oncogene* **18,** 2557–2566
32. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) *Nat. Cell. Biol.* **1,** 193–199
33. Hadvary, P., Sidler, W., Meister, W., Vetter, W., and Wolfer, H. (1991) *J. Biol. Chem.* **266,** 2021–2027
34. Hauptman, J. B., Jeunet, F. S., and Hartmann, D. (1992) *Am. J. Clin. Nutr.* **55,** 309S–313S
35. Rank, K. B., Evans, D. B., and Sharma, S. K. (2000) *Biochem. Biophys. Res. Commun.* **271,** 469–473
36. Pizer, E. S., Wood, F. D., Pasternack, G. R., and Kuhajda, F. P. (1996) *Cancer Res.* **56,** 745–751
37. Schulman, B. A., Carrano, A. C., Jeffrey, P. D., Bowen, Z., Kinnucan, E. R., Finnin, M. S., Elledge, S. J., Harper, J. W., Pagano, M., and Pavletich, N. P. (2000) *Nature* **408,** 381–386
38. Yang, G., Ayala, G., Marzo, A. D., Tian, W., Frolov, A., Wheeler, T. M., Thompson, T. C., and Harper, J. W. (2002) *Clin. Cancer Res.* **8,** 3419–3426